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(54) Title: GENOMIC MISMATCH SCANNING		
(57) Abstract Genetic mapping is provided by either of two related general procedures. In the first procedure, mapping is provided by identifying genetic regions from which DNA fragments derived from two individuals can combine to form extensive hybrids free of base mismatches. DNA is processed by a method that allows perfectly-matched hybrid DNA molecules formed between DNAs from the two individuals, to be separated from imperfectly-paired DNA hybrids or hybrids in which both strands are from the same individual's DNA. The perfectly-matched hybrid DNAs can then be labeled and the labeled DNA used as probes to identify loci of indentity-by-descent between the two individuals. In the second prodecure, nicks are introduced specifically into DNA hybrids formed between non-identical alleles from a region of heterozygosity in an individual diploid genome. The nicked DNA molecules are then specifically labeled to provide probes for identifying regions of heterozygosity in the genome of an indi- vidual.		

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GENOMIC MISMATCH SCANNING

CROSS-REFERENCE TO GOVERNMENT GRANT

This invention was made with Government support under contract HG00450 awarded by the National Institutes of Health. The government may have certain rights in this invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application serial no. 880,167 filed on May 6, 1992.

10

INTRODUCTION

Technical Field

The field of this invention is genetic mapping.

Background

Linkage mapping of genes involved in disease susceptibility and other traits in humans, animals and plants has in recent years become one of the most important engines of progress in biology and medicine. The development of polymorphic DNA markers as landmarks for linkage mapping has been a major factor in this advance. However, current methods that rely on these markers for linkage mapping in humans are laborious, allowing screening of only at most a few markers at a time. Furthermore, their power is limited by the sparsity of highly-informative markers in many parts of the human genome.

25

To map genes whose manifestations are recognized only in the whole organism, the standard approach relies on identifying linkage between the trait and a genetic marker whose map position is already known. The most abundant and generally useful class of markers in the human genome are DNA sequence polymorphisms—either restriction fragment length polymorphisms (RFLP's) or other DNA polymorphisms that can be detected by hybridization with specific probes or by amplification using specific primers.

RFLP mapping and related methods have had dramatic successes. However, their utility is limited by two problems: First, many discrete loci need to be examined, but only one or a few loci can be typed at a time, which procedure is arduous; second, the low density on the map and low polymorphism information content (PIC value) of available markers means that multiple members of each family need to be typed to obtain useful linkage information, even when only two share the trait of interest. While these disadvantages can be overcome to some degree by automation and technical improvements, as well as by developing closely-spaced extremely polymorphic markers, the use of discrete markers for specific map intervals has inherent limitations. The limitations are particularly marked when applied to mapping strategies that seek to reduce the number of individuals that need to be analyzed in each family.

It is generally much easier to collect many pairs of related individuals who share a trait of interest than to collect a few large, well-documented pedigrees in which the trait is segregating. In the former case, the absolute number of individuals is smaller. For medically-significant traits, affected individuals are likely to present themselves for examination, whereas other family members need to be traced and recruited. Furthermore, individuals vital to pedigree analysis may be deceased. Yet the low density and low information content of available markers makes the use of pedigrees almost mandatory for linkage mapping by RFLP analysis.

Collection of appropriate families frequently poses the principal barrier to mapping genes that influence human traits, particularly genetically complex traits.

Strategies have been reported for linkage mapping using
5 the information in DNA from multiple very small sets of affected relatives (typically pairs or even single individuals). However, these strategies depend upon the availability of closely-spaced highly information genetic markers throughout the genetic map.

10 The issues raised above in reference to linkage mapping also apply to genetic risk assessment in medicine.

In principle, any base that differs among allelic sequences could serve as a marker for linkage analysis. Single-base differences between allelic single copy
15 sequences from two different haploid genomes have been estimated to occur about once per 300 bp in an outbred Western European population. This calculates to a total of about 10^7 potential markers for linkage analysis per haploid genome. Only a tiny fraction of these nucleotide
20 differences contribute to mapping using current methods. There is, therefore, substantial interest in developing new methods that utilize the available genomic information more efficiently and can provide information concerning multi-gene traits. Such methods could be valuable, not
25 only for gene mapping, but also for genetic diagnosis and risk assessment.

Relevant Literature

Articles describing the use of RFLP's are described in Botstein, et al., (1980) Am. J. Hum. Genet. 32:314-331;
30 Donis-Keller, et al. (1988) Cell 51:319-337; Kidd, et al. (1989) Cytogenet. Cell. Genet. 51:622-947 and Risch (1990) Am. J. Hum. Genet. 46:242-253. Mapping strategies may be found in Risch (1990) Am. J. Hum. Genet. 46:229-241;
Lander and Botstein (1987) Science 236:1567-1570; and
35 Bishop and Williamson (1990) Am. J. Hum. Genet. 46:254-265. Sandra and Ford, (1986) Nucleic Acids Res.

14:7265-7282 and Casna, et al. (1986) Nucleic Acids Res.
14:7285-7303 describe genomic analysis.

SUMMARY OF THE INVENTION

Genomic analysis is achieved through the process of:
5 Digesting DNA to be compared from two different sources,
usually individuals who are genetically related or
suspected of being genetically related, with a restriction
enzyme that cuts relatively infrequently; combining single
strands of the genomic fragments from the two individuals
10 under conditions whereby heterohybrids (hybrids containing
one strand from each individual) can be distinguished from
homohybrids (hybrids containing both strands from the same
individual); separating homohybrids from heterohybrids;
separating mismatch-free heterohybrids from hybrids with
15 mismatches; preparing labeled probes from the mismatch-
free heterohybrids; and identifying regions of genetic
identity between the two individuals by means of said
labeled probes. The mismatch-free heterohybrids provide
~~highly specific probes for regions of genetic identity by~~
20 descent, since sufficiently large hybrid DNA molecules
formed from non-identical regions are expected to have at
least one and usually many base mismatches.

Alternatively, one may map regions of heterozygosity,
and, by inference, homozygosity in a single individual by
25 isolating DNA fragments substantially free of multicopy
DNA; melting the DNA and reannealing to provide for hybrid
fragments; introducing nicks specifically in mismatched
DNA; labeling the nicked DNA; and using the labeled DNA as
probes to identify regions of heterozygosity. Regions of
30 homozygosity or hemizygosity (where all or a significant
portion of a chromosome is missing, e.g. aneuploidy) are
inferred by the absence of hybridized label.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods and compositions are provided for genomic
35 mapping by identifying regions of the genome at which DNA
sequences from two DNA sources are perfectly identical

over long stretches (typically 10^3 to 2×10^4 nt). Depending upon the nature of the probe, the procedures may vary. In a procedure that allows for labeling of regions of genetic identity between two individuals, DNA sources
5 are digested with a restriction enzyme that cuts infrequently; the resulting DNA is processed to isolate mismatch-free heterohybrid dsDNA fragments free from mismatch containing or homohybrid fragments; the mismatch-free heterohybrid fragments are labeled and then used to
10 identify regions of genetic identity between the two sources.

Alternatively, to map regions of homozygosity or heterozygosity in an individual genome, one may digest the DNA from that individual; optionally, remove multi-copy
15 DNA; melt and reanneal the DNA; introduce nicks into mismatched dsDNA; label the mismatched dsDNA; and use the labeled DNA as probes for identifying genomic regions that are heterozygous, leaving regions that are homozygous or hemizygous having substantially lower labeling.

20 The DNA source may be any source, haploid to polyploid genomes, normally eukaryotic, and may include vertebrates and invertebrates, including plants and animals, particularly mammals, e.g. humans. The DNA will be of high complexity where each of the sources will
25 usually have greater than 5×10^4 bp, usually greater than 10^6 bp, more usually greater than about 10^7 bp. Thus, in any situation where one wishes to compare two sources of DNA as to their genetic similarities, whether the sources are related or not, the subject method may be employed.
30 Usually, the sources will be related, being of the same species, and may be more closely related in having a common ancestor not further away than six, frequently four, generations.

For linkage mapping or genetic diagnosis, genetically
35 related individuals are required. Thus, the subject method may find application in following segregation of traits associated with breeding of plants and animals, the association of particular regions in the genomic map with

particular traits, especially traits associated with multiple genes, the transmission of traits from ancestors or parents to progeny, the interaction of genes from different loci as related to a particular trait, and the like. While only two sources may be involved in the comparison, a much larger sampling may be involved, such as 20 or more sources, where pairwise comparisons may be made between the various sources. Relationships between the various sources may vary widely, e.g. grandparents and grandchildren; siblings; cousins; and the like.

Depending upon whether the regions of genetic identity or regions of non-identity are to be labeled, the treatment of the DNA from the source will vary. The DNA may be processed initially in accordance with conventional ways, lysing cells, removing cellular debris, separating the DNA from proteins, lipids or other components present in the mixture and then using the isolated DNA for restriction enzyme digestion. See Molecular Cloning, A Laboratory Manual, 2nd ed. (eds. Sambrook et al.) CSH Laboratory Press, Cold Spring Harbor, NY 1989. Usually, at least about 0.5 μg of DNA will be employed, more usually at least about 5 μg of DNA, while less than 50 μg of DNA will usually be sufficient.

The following procedure will address solely the methodology employed for isolating and labeling of DNA corresponding to regions of identity-by-descent between two related individuals. The total DNA from both cellular sources is digested completely with a restriction enzyme that cuts relatively infrequently, generally providing fragments of strands of about $0.2-10 \times 10^4$ nt, preferably of about $0.5-2 \times 10^4$ nt. The size is selected to substantially ensure the presence of at least one GATC sequence, and at least one base difference between any allelic fragments not identical by descent i.e. to ensure that homohybrid fragments, or heterohybrid fragments that are not identical by descent, sustain at least one cut in a subsequent step. This enzyme will normally recognize at least a 6-nucleotide consensus sequence and may involve

either blunt-ended or staggered-ended cuts. For the method described in detail here, an enzyme that cuts to leave a protruding 3' end is needed. The protruding 3' ends are preferred specifically when exonuclease III digestion, followed by BNDC binding, is ultimately used to eliminate homohybrids and mismatched DNA's. Restriction enzymes yielding other sorts of ends may be preferred if other specific steps are substituted, as described further below.

10 The resulting DNA fragments are then processed to provide for a means of separating complementary DNA hybrids, where the two strands are from different sources, from complementary DNA hybrids where the two strands are from the same source. This may be achieved in different
15 ways. The method exemplified in the subject invention uses the following steps. DNA from one of the sources is methylated with a sequence specific methylase, such as Dam methylase or a restriction methylase, so as to substantially completely methylate the consensus sequences
20 of the DNA from one of the sources. The other source is left unmodified or methylated completely with a different restriction methylase.

The two DNA samples are then mixed, denatured and allowed to reanneal. A practical rate of complete
25 annealing of the complex DNA samples can be achieved by using chemical or protein catalysts under conditions that preserve large DNA strands (Casna, et al. (1986) Nucleic Acids Res. 14:7285-7303; Barr and Emanuel (1990) Anal. Bioch. 186:369-373; Anasino (1986) *ibid* 152:304-307). It
30 is also necessary to avoid or minimize network formation resulting from rapid hybridization of non-allelic repeated sequences, so that simple fully-duplex products can be recovered even when dispersed repetitive sequences are embedded in them. Annealing conditions that have been
35 shown to meet this requirement are FPERT conditions as described in Casna (1986) supra.

The reannealed DNA mixture is digested with two methylation-sensitive restriction endonucleases. Hybrids

formed between the two different DNA specimens will be hemimethylated at the methylation sites. The restriction enzymes are selected so as to digest sites which are unmethylated or doubly methylated, while being incapable of cleaving the hemimethylated sites. Desirably, the sequence will be a relatively common sequence, generally occurring on the average of about $1-10 \times 10^2$, preferably about $1-5 \times 10^2$. A more stringent selection for mixed-donor hybrids can be achieved by using additional combinations of restriction/modification enzymes (Casna (1986) *supra*). Optionally, one may remove unannealed DNA (single stranded DNA) at this time using any convenient method, e.g. adsorption to BNDC.

Various combinations of enzymes can be employed. The combination should ensure that there is at least one cut in any homohybrid fragment, preferably at least two or more, so the sequence should be relatively common. For example, *E. coli* *dam* methylase, which recognizes GATC for methylation, may be employed for methylation. This modification enzyme may then be used with the methylation-sensitive restriction endonucleases *DpnI* and *MboI*, which cleave at GATC sites, the former at doubly-methylated sites and the latter at unmethylated sites. The particular sequence "GATC" is found every few hundred bp in the human genome. While a single restriction/modification site is preferable, two or three sites may be involved where different combinations of modification and restriction enzymes are employed. With DNA from sources other than human, other combinations of modification enzymes and restriction enzymes may be employed.

An alternative procedure is as follows. By cutting the two DNA samples using a different restriction enzyme for each--specifically two enzymes that share a common recognition sequence but in one case cut with an N-base 3' overhang, and in the other with an N-base 5' overhang--only the heterohybrids will have flush ends. An example of such a pair of restriction enzymes for the case N=4, is

Acc65I and KpnI. The hybrids with both strands derived from the same DNA sample will retain either a 5' or 3' overhanging end.

The flush-ended heterohybrids will uniquely be able to be ligated to a flush-ended partner, such as an oligonucleotide. This oligonucleotide might, for example, have a hairpin structure, such that the "capped" ends are protected from exonuclease digestion. Variations on this principle are possible, exploiting the distinctive structures of the ends of heterohybrids compared with homohybrids, when related restriction enzyme pairs are used.

This strategy for selecting heterohybrids can replace the MboI/DpnI digestion step in selecting heterohybrids. However, a 5' to 3' exonuclease, such as bacteriophage lambda exonuclease, or a combination of a helicase and exonuclease VII and/or I, or another combination of enzymes to allow digestion of all uncapped ends would need to be used in addition to, or in place of exonuclease III, following the MutHLS nicking step in the procedure outlined below.

Other techniques may also be used for the initial separation of homohybrids from heterohybrids. By growing the cells from one of the sources with heavy isotope-labeled nucleosides, heavy atom labeled nucleosides, or other isotope labeled precursors, the two strands from the different sources will differ in density. Isotopes that may be used include ^{15}N , ^{13}C , ^2H , etc. The duplexes may then be separated by density banding.

Alternatively, one may label the DNA from the two sources with different labels, e.g. using labeled nucleotides and terminal deoxynucleotidyl transferase, by random conjugation, and the like. One can then separate all of the duplexes as to one label, and then divide that group into homo- and heterolabeled duplexes. For example, biotin and avidin may be used to separate at one stage, where the avidin is bound to magnetic beads, and 2,4-dinitrophenyl and anti-(2,4-dinitrophenyl) may then be

used for the second separation, where the anti-(2,4-dinitrophenyl) is bound to a support.

Alternatively, one may select restriction enzymes which provide for overhangs, where heterohybrids will
5 result in blunt ends or overhangs that differ from those of the homohybrids. One may then use the overhangs to separate homohybrids leaving the heterohybrids.

Returning to the description of a principal embodiment, the resulting mixture of uncut heterohybrids,
10 and MboI or DpnI cut homohybrids is then subjected to a system which allows for separation of DNA duplexes with some mismatched base pairs from complementary perfectly-matched DNA duplexes. See, for example, Lahue, et al. (1989) Science 245:160-164; Su and Modrich (1986) Proc. Natl. Acad. Sci. USA 83:5057-5061; Grilley, et al. (1989)
15 J. Biol. Chem. 264:1000-1004; Su, et al. (1989) Genome 31:104-111; and Learn and Grafstrom (1989) J. Bacteriol. 171:6473-6481.

Illustrative of such a system is the "methyl-directed
~~20 mismatch-repair-pathway of *E. coli*~~." The system uses the purified mutS, mutL, mutH and uvrD gene products, as well as exonuclease I, exonuclease VII or RecJ exonuclease, single strand binding (SSB) protein and DNA
polymerase III. In vitro, seven of the eight possible
25 single base mismatches, as well as small insertions and deletions are efficiently recognized. When DNA synthesis is prevented, the system specifically introduces large gaps in the mismatch-containing molecules. The purified mutS, mutL and mutH proteins act in concert to introduce
30 nicks specifically into DNA molecules that contain base mismatches. With the exception of C-C mismatch, the other mismatches are effectively identified by one or more of the MutX enzymes (X indicates S, L or H).

Exonuclease III can initiate exonucleolytic digestion
35 at a nick and digest the nicked strand in the 3' to 5' direction to produce a gap. Exonuclease III can also initiate digestion at a recessed or flush 3' end, but it cannot initiate digestion at a protruding 3' end of a

linear duplex. Digestion from the ends of the linear DNA hybrids can, therefore, be prevented by choosing a restriction enzyme that produces protruding 3' ends for the initial digestion of the genomic DNA (Henikoiff (1984)

5 Gene 28:351-360). The DNA ends produced by the restriction enzymes used to make the smaller fragments for distinguishing hemimethylated sites from unmethylated or dimethylated sites are selected to provide recessed (e.g. MboI) or flush (DpnI) 3' termini and these termini are
10 susceptible to digestion by exonuclease III. Therefore, exonuclease III provides for partial single strands in hybrid molecules where both strands are derived from the same individual or where the strands contain base mismatches.

15 In carrying out the process, the duplexes obtained from annealing and restriction digestion are exposed to the methyl-directed mismatched repair system *in vitro*. The mutS, mutL and mutH introduce nicks specifically into the mismatched DNA molecules, while the exonuclease III
20 can introduce gaps at nicked sites and at recessed 3' termini. The mismatch-free heterohybrids, corresponding to regions of identity-by-descent between the sources, can now be distinguished from all other duplexes by virtue of the absence of significant gaps or partial single strand
25 regions.

The partially single stranded and single stranded DNA is now separated from the fully-duplex DNA. This can be efficiently achieved using benzoylated naphthylated DEAE cellulose (BNDC). At high salt concentration, BNDC
30 retains single stranded and partially single-stranded DNA molecules with high efficiency and may be separated by centrifugation or other separation means. The unbound DNA molecules are recovered, which in this case comprise complementary sequences from the two sources.

35 A more complete methyl-directed mismatch repair enzyme system may ultimately prove to be superior in specificity to the simple system using only MutS, MutL and MutH. For example, MutL, MutS and MutH, plus helicase II

(UvrD protein), exonuclease I, exonuclease VII, single-strand binding protein, and DNA polymerase III, acting in concert, can carry out mismatch dependent DNA synthesis, and thereby specifically introduce labeled or modified nucleotides into mismatch-containing DNA molecules. For example, by using biotinylated nucleotides, mismatched DNA molecules can be specifically biotinylated, and then immobilized avidin or related methods can be used to remove the mismatched molecules from a mixture of perfectly-matched and mismatched DNA molecules. Alternatively, use of the same set of enzymes, excluding DNA polymerase, would lead directly to gaps in the mismatched DNA eliminating the exonuclease III step. For such a procedure, the ends of the linear DNA molecules produced in the initial annealing step would need to be protected from the action of the exonucleases and helicase. This procedure would thus interface well with the end-capping method suggested above as an alternative method for selection for heterohybrid molecules.

~~20 It is fairly obvious that related enzyme systems~~
based on mismatch repair protein from E. coli or other organisms could in principle substitute here for the particular enzyme system described.

The mismatch-free heterohybrid dsDNA duplexes may be used without expansion. The subject method provides a sufficiently clean separation of the mismatch-free heterohybrid dsDNA duplexes in sufficient amount to allow for their labeling and direct use as probes. By using a readily available amount of DNA which can be efficiently handled, generally from about 0.5 to 100 μ g DNA, usually about 1 to 10 μ g DNA, from each source, a satisfactory amount of the mismatch-free heterohybrid dsDNA duplexes are obtained for labeling and probing a DNA sample.

The mismatch-free heterohybrid dsDNA sequences from the two sources can be used for identifying the regions of identity-by-descent between the two sources. Conveniently, the dsDNA may be labeled for use as a probe to identify the corresponding genomic regions. A wide

variety of labels may be employed, particularly radio-isotopes, fluorescers, enzymes, and the like. The particular choice of label will depend upon the desired sensitivity, the nature of the genomic sample being probed, the sensitivity of detection required, and the like. Thus, the more complex the genome under analysis, the higher the sensitivity which would be desired. Various instruments are available which allow for detection of radioactivity, fluorescence, and the like.

10 The probes may be prepared by any convenient methodology, such as nick translation, random hexamer primed labeling, polymerase chain reaction using primers that prime outward from dispersed repetitive sequences or random sequences, and the like.

15 The DNA that is probed may take a variety of forms, but essentially consists of a physically-ordered array of DNA sequences that can be related back to the physical arrangement of the corresponding sequences in the genome (See Boyle, et al. (1990) Genomics 7:127-130; Penkel, et al. (1988) PNAS USA 87:6634-6638). A metaphase chromosome spread is one naturally-occurring example of such an array. Alternatively, and preferably, a partial or a complete collection of cloned, amplified, or synthetic DNA sequences corresponding to known genetic locations, immobilized in an ordered array on a solid substrate such as a membrane or a silicon or plastic chip, can be used as the target for probing.

25 The hybridizations with the probes are performed under conditions that allow the use of complex mixtures of DNA probes and suppress artifactual hybridization to repeated sequences (Boyle, et al. (1990) Genomics 7:127-133; Pinkel, et al. (1988) Proc. Natl. Acad. Sci. USA 85:9138-9142; Lichter, et al. (1990) ibid 87:6634-6638). For example, in the case of grandparent-grandchild pairs, hybridization should occur in approximately 25 large patches (averaging about 4 μ in length when prometaphase chromosomes are used), which in aggregate should cover about one-half of the genome.

The boundaries of the patches will be determined by the ends of chromosomes and sites of meiotic crossing over. The boundaries will reflect sites of meiotic recombination that occurred in meioses intervening between the two relatives. Since the areas of hybridization will typically be in large contiguous patches, the method is very robust with respect to contamination of the probe with sequences representing regions that are not identical between the two subjects. Even if only a modest enrichment of identical-by-descent sequences is achieved, the patches of identity and non-identity should be distinguishable as contiguous blocks of greater or lower signal intensity.

Alternatively, rather than using the selected mismatch-free, heterohybrid restriction fragments as probes, they themselves can be immobilized on a solid substrate, typically a Southern blot performed after resolving the DNA restriction fragments by gel electrophoresis. The immobilized fragments can then be probed by hybridization using labelled DNA probes specific to regions of interest. The presence or absence of hybridizing bands recovered from a specific pairwise comparison will indicate whether the pair has identity by descent at the locus in question. This procedure is likely to be useful for refining the resolution of a map, after initial mapping is achieved by using selected fragments as probes.

Genetic regions identified by this mapping method may be cloned by standard methods or in some cases by direct cloning of the sequences selected by genomic mismatch scanning. These sequences can then be analyzed for their biological function, and in some cases used directly or in synthetic or modified form for diagnostic or therapeutic applications.

When the region of genetic identity between two or more individuals is sufficiently small, for example, in plant breeding when products of serial backcrosses with selection for a useful trait are compared, it may be

useful to clone the selected identical-by-descent restriction fragments, since the resulting clone pool would be highly enriched for the desired gene sequence (responsible for the selected trait).

- 5 An alternate embodiment of genetic mapping looks to differences within an individual genome. One may look to identify regions of a genetic map where an individual or a sample is or is not heterozygous. This method is predicated on the isolation of single-copy sequences
- 10 corresponding to regions of heterozygosity in the test individual, based on the ability of that individual's DNA to give rise to hybrid DNA molecules with base mismatches when the individual's DNA is denatured and reannealed. The selected mismatched hybrid sequences are then labeled
- 15 and used as probes for hybridization to a physically ordered array of a genomic DNA sample. Because regions of the genome that lack heterozygosity are unable to produce single-copy DNA hybrids with base mismatches, these regions are visualized as gaps in the hybridization
- 20 pattern. The following is an exemplary protocol.

- The DNA sample is digested to completion with a restriction enzyme that cuts the DNA frequently enough that most of the resulting fragments should not contain repetitive sequences. Illustrative restriction enzymes
- 25 include enzymes having four nucleotide consensus sequences, such as AluI, RsaI, TaqI, HaeIII and MboI. The resulting fragments will for the most part be in the range of about 200-4000 nucleotides. After melting or denaturing the DNA, the DNA is allowed to reanneal in
- 30 solution, where the rapidly reannealing multi-copy or repeated DNA sequences (low C_0t) are removed, for example, by hydroxyapatite chromatography, based on their rapid reannealing, followed by allowing the remaining DNA to anneal completely. Removal of the low C_0t number DNA is
- 35 desirable, but may not be essential. After complete reannealing, desirably removing residual unannealed DNA, the small, mostly single-copy DNA fragments that remain are incubated with the methyl-directed mismatch repair

proteins described above and ATP to produce nicks in mismatched DNA duplexes. The nicks introduced specifically in mismatched molecules by the methyl-directed mismatch repair allow for nick-translation DNA synthesis by the DNA polymerase, and thus allow labeling with radiolabeled or other labeled nucleotide triphosphates. A polymerase that lacks 3' to 5' exonuclease activity, but retains 5' to 3' exonuclease activity, e.g. Taq polymerase, is preferred for this step to avoid labeling by replacement synthesis at the ends of the fragments.

The described procedure will provide probes that densely and specifically cover the regions of the genetic map that are heterozygous in the test individual. Conversely, the regions that are not heterozygous will not provide labeled probes and so should be recognized as distinctive gaps in the hybridization signal. In general, except where the coefficient of inbreeding is very low, the regions of homozygosity will be in contiguous patches of sufficient size that even a few, e.g. 3-10-fold difference in hybridization intensity between homozygous and heterozygous sites should produce discernible boundaries. Thus, as indicated previously, the separation of heterozygous from homozygous sequences need not be absolute. In addition to clinical and mapping applications, this procedure is likely to be useful in plant and animal breeding, since backcrossing and selection can be used to isolate a gene responsible for a trait of interest to a small region of heterozygosity or homozygosity.

For convenience, kits may be supplied which provide the necessary reagents in a convenient form and together. For example, for the genomic mismatch screening, kits could be provided which would include at least two of the following: The restriction enzymes: one or more which provide for average fragments from the target genome of a size in the range of about $0.5 - 10 \times 10^4$; one or more modification enzymes; and restriction enzymes which

distinguish between hemimethylation and unmethylated or dimethylated consensus sequences; enzymes capable of introducing nicks at mismatches and expanding the nick to a gap of many (≥ 10) nucleotides; DNA polymerase; BNDC
5 cellulose; and labeled triphosphates or labeled linkers for blunt end ligation or other composition for labeling the sequences to provide probes. Other components such as a physically ordered array of immobilized DNA genomic clones or metaphase chromosomes, automated systems for
10 determining and interpreting the hybridization results, software for analyzing the data, or other aids may also be included depending upon the particular protocol which is to be employed.

The subject methodology may find particular
15 application in mapping genes by use of affected relative pairs--that is, pairs of relatives that have a genetically influenced trait of interest. "Affected relative pair" methods are preferred, particularly when the penetrance of the allele that confers the trait is low or age-dependent,
20 or when the trait is multigenic or quantitative, e.g. height and build. Disease-susceptibility genes are particularly relevant. By determining where on the genetic map a small set, including two, of "affected" relatives have inherited identical sequences from a common
25 source, and disregarding other family members, a highly efficient strategy for extracting linkage information from a pedigree is provided. The resulting identity-by-descent maps from multiple pairs of similarly-affected relatives can be combined and the composite map searched for loci
30 where genotypic concordance between affected relatives occurs more frequently than would be expected by chance. With a sufficiently large number of affected relative pairs, such an analysis can reveal the positions of genes that contribute even a slight susceptibility to the trait.
35 The procedure may also find wide application in routine screening for shared genetic risks in families.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Saccharomyces cerevisiae, Baker's yeast, was used as a test system, because this is genetically the best characterised eukaryotic organism, and it is easy to prepare and characterize yeast clones of defined genetic relatedness.

Test of the method: Two independently isolated haploid clones of Saccharomyces, Y55 (HO his4 leu2 CAN1^S ura3 GAL2) and Y24 (ho HIS4 LEU2 MATa can1^R URA3 GAL2), both derivatives of common lab strains, were used for the experiment. We estimate from RFLP analysis that Y55 and Y24 (a derivative of S288C) differ at approximately one base pair per 100. The two strains were mated, and the resulting diploid hybrid was sporulated, yielding 4 haploid spore clones. For any given genetic locus, each spore clone ("daughter") received either the Y55 or the Y24 allele. The purpose of the test was to determine if we could specifically isolate, en masse, DNA from all the loci at which two individuals (here, pairs of parent and daughter clones) share genetic identity, excluding DNA from all regions where there was no identity by descent between the pair. We applied our genome mismatch scanning method to determine for several loci, which spore clones had identity by descent with each parent. The results of the genome mismatch scanning analysis were compared with results from conventional analysis (Table 1), using auxotrophic and drug resistance markers. Conventional analysis consisted of testing for growth on appropriate selective media. Four loci were tested--HIS4, CAN1, URA3, GAL2. HIS 4 is on chromosome 3, CAN1 and URA3 on chromosome 5, and GAL2 is on chromosome 12. Our analysis of these loci included a total of 15 independent PstI restriction fragments, each of which constituted an independent test of the genomic mismatch scanning method, as their sometimes adjacent location in the genome was immaterial to their behaviour in the selection. The result of the test was that all 15 PstI restriction fragments analyzed were recovered if and only if they were

identical by descent between the parent and daughter being compared (Tables 1 and 2). This result confirms the principles underlying genomic mismatch scanning.

Procedure:

5 1. DNA Isolation: High molecular weight DNA was isolated from each yeast strain (parent or spore clone) by a standard method (Methods in Enzymology 194 (chapter 11):169-182).

10 2. Initial Restriction Enzyme Digestion: Each DNA sample was digested completely with PstI restriction enzyme. The DNA was recovered by phenol:chloroform extraction, and ethanol precipitation, and resuspended in Tris-HCl 10 mM EDTA 1 mM, pH 8.0.

15 3. Methylation of DNA From Parental Strains with Dam Methylase: DNA samples from the two parental strains, Y55 and Y24, were fully methylated with E. coli Dam methylase (New England Biolabs), at a DNA concentration of 0.25 mg/ml, using 4 units of enzyme/ μ g of DNA in an overnight incubation at 37°, in the buffer recommended by
20 the manufacturer. The samples were extracted with phenol:chloroform, ethanol precipitated, and resuspended in Tris-HCl 10 mM, EDTA 1 mM, pH 8.0.

4. Mixing and Solution Hybridization of Paired Test DNA Samples:

25 A. Y55 DNA (5 μ g in 45 μ l) + spore clone 1b DNA (5 μ g in 80 μ l) was denatured by adding 7.5 μ l of 5M NaOH. After 10 min at room temperature, the sample was neutralized by adding 16 μ l of 3 M MOPS acid. 32 μ l of formamide and 200 μ l of 2X PERT buffer (4M NaSCN 20 mM
30 Tris-HCl pH 7.9, 0.2 mM EDTA) were then added, and the sample was adjusted to 400 μ l with water. 90% phenol in water was added until an emulsion was apparent (about 80 μ l), and then the sample was agitated to maintain the emulsion for 12 hours at room temperature (typically about
35 23°).

B. Y55 DNA (5 μ g in 45 μ l) + spore clone 1c DNA (5 μ g in 45 μ l) was denatured by adding 5.4 μ l of 5M NaOH. After 10 min at room temperature, the sample was

neutralized by adding 12 μ l of 3 M MOPS acid. 32 μ l of formamide and 200 μ l of 2X PERT buffer were then added, and the sample was adjusted to 400 μ l with water. 90% phenol in water was added until an emulsion was apparent
5 (about 150 μ l), and then the sample was agitated to maintain the emulsion for 12 hours at room temperature (typically about 23°).

C. Y24 DNA (5 μ g in 45 μ l) + spore clone 1a DNA (5 μ g in 45 μ l) was denatured by adding 5.4 μ l of 5M NaOH.
10 After 10 min at room temperature, the sample was neutralized by adding 12 μ l of 3 M MOPS acid. 32 μ l of formamide and 200 μ l of 2X PERT buffer were then added, and the sample was adjusted to 400 μ l with water. 90% phenol in water was added until an emulsion was apparent
15 (about 150 μ l), and then the sample was agitated to maintain the emulsion for 12 hours at room temperature (typically about 23°).

D. Y24 DNA (5 μ g in 45 μ l) + spore clone 1c DNA (5 μ g in 45 μ l) was denatured by adding 5.4 μ l of 5M NaOH.
~~20 After 10 min at room temperature, the sample was~~
neutralized by adding 12 μ l of 3 M MOPS acid. 32 μ l of formamide and 200 μ l of 2X PERT buffer were then added, and the sample was adjusted to 400 μ l with water. 90% phenol in water was added until an emulsion was apparent
25 (about 150 μ l), and then the sample was agitated to maintain the emulsion for 12 hours at room temperature (typically about 23°).

To recover DNA, the samples were each extracted once with chloroform, then ethanol precipitated, and
30 resuspended in 200 μ l of Tris/EDTA.

5. Digestion of Homohybrid Molecules (Both Strands From the Same Source) with DpnI+ and MboI+: 105 μ l of each of the homohybrid strands was digested at 37° for 2 hours in a final volume of 400 μ l of NEB buffer 3 with 100
35 units of DpnI and 25 units of MboI.

6. Removal of Residual Unannealed DNA: After MboI/DpnI digestion, samples were extracted with phenol/chloroform, 100 μ l of 5M NaCl was added to each and

then samples were incubated with 100 mg of BNDC cellulose (Sigma) equilibrated with 50 μ M tris, pH 8.0, 1M NaCl, at 4° for 3 hours. The sample was centrifuged at 14000 rpm in a microfuge, then the supernatant was extracted twice
5 with phenol/chloroform and once with chloroform, then ethanol precipitated, washed with 70% ethanol, dried and resuspended in 90 μ l of Tris-HCl 10 mM EDTA 1 mM, pH 8.0.

7. Selective nicking of mismatched hybrid DNA's:
15 μ l of each DNA sample was mixed with 5.2 ng of Muth protein, 340 ng of MutL protein, 700 ng of MutS protein (all proteins provided in purified form by Paul Modrich, Duke University), in a final volume of 60 μ l of a buffer consisting of: 50 mM Hepes (pH 8.0), 20 mM KCl, 5 mM MgCl₂, 1 mM DTT, 50 μ g/ml bovine serum albumin, and 2 mM
15 ATP. The mixture was incubated at 37° for 30 minutes, and the reaction was then stopped by heating to 65° for 10 minutes.

8. Exonuclease III Digestion to Convert Nicks into Single-Strand Gaps, and Ends from MboI or DpnI Cleavage
20 into Single-Strand Tails: The volume of the entire sample from step 7 was adjusted to 200 μ l by adding 140 μ l of a buffer consisting of: 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 10 mM β -mercaptoethanol. Then 10 units of exonuclease III were added and incubation continued for 10
25 min at 37°. This reaction was stopped by adding EDTA to 10 mM, followed by extraction with phenol/chloroform.

9. Removal of Partially or Fully Single-Stranded DNA Molecules from the Mixture: 50 μ l of 5M NaCl + 250 μ l of 1M NaCl were added to adjust to a volume of 500 μ l at a
30 concentration of 1 M NaCl. 100 mg of BNDC cellulose equilibrated with 50 mM tris, pH 8.0, 1M NaCl, was added and the mixture incubated at 4° for 3 hours. (Sedert, et al. (1967) J. Mol. Biol. 26:537-540; Iyer and Rupp (1971) Bioch. Biophys. Acta. 228:117-126) The mixture was
35 centrifuged at 14000 rpm for 1 min, then the supernatant was extracted once with phenol/chloroform, and ethanol precipitated overnight. The small pellets were resuspended in 15 μ l of Tris-HCl 10 mM, EDTA 1 mM, pH 8.0.

10. Analysis of the Selected DNA pool by Southern Blotting: One-sixth of each of the resulting DNA samples were electrophoresed through a 0.7% agarose gel, in TBE buffer for 15 hours at 70 volts. DNA was transferred to a nylon filter by Southern blotting, and the filter was probed successively with labelled DNA from lambda phage clones corresponding to the 4 specific genetic loci, HIS4, CAN1, URA3 AND GAL2. In each case, 3-5 restriction fragments were readily detected in the lanes corresponding to DNA samples that had identity by descent at the test loci, and not in the lanes corresponding to samples that were known from direct tests not to match at the locus being probed (see Table 1 and 2).

15 Table 1.

		Locus			
Strain		CAN1	URA3	HIS4	GAL2
parents	Y24	A	A	A	A
	Y55	B	B	B	B
daughters	1a	B	B	A	A
	1b	A	B	A	A
	1c	A	A	B	B

- 20 The two alleles at each locus are designated A and B, respectively for the alleles present in Y24 and Y55. Each spore clone inherits an allele from one of its two parents, either the A allele from Y24 or the B allele from Y55. The alleles at these loci can be distinguished directly by testing for growth in specific media.

25

Table 2. Summary of the results of the Genome Mismatch Scanning test.

Comparison #	Relative Pair	LOCUS			
		CAN1	URA3	HIS4	GAL2
1	Y24/daughter 1a	-	-	+	+
2	Y55/daughter 1c	-	-	+	+
3	Y24/daughter 1c	+	+	-	-
4	Y55/daughter 1b	-	+	-	-
number of restriction fragments		[4]	[4]	[3]	[5]

10 "-" indicates no DNA was recovered for any of the restriction fragment bands detected by the DNA probe specific for the indicated locus (neglecting faint bands from cross-hybridization to unlinked sequences).

15 "+" indicates recovery of DNA in all restriction bands detected by the DNA probe specific to the indicated locus.

The number of restriction fragment bands surveyed by the probe used for the indicated locus is indicated in brackets in the bottom row of the table. The probes used in each case were bacteriophage lambda clones from the ordered collection established by Maynard Olsen. The specific clones used as probes were: CAN1: clone 5917, HIS4: clone 4711, URA3: clone 6150, GAL2: clone 6637. The clone numbers are the numbers assigned by Maynard Olsen. For convenience, only 4 of the eight possible parent daughter combinations were tested. The results of the genetic tests for 4 loci are shown in Table 1. Numerous other pairwise comparisons have subsequently been tested with similar results.

30 It is evident from the above results, that the subject methodology provides for numerous advantages. The methods provide access to a large set of highly polymorphic markers required for linkage mapping with small family units. A great increase in the effective number of informative markers is achieved without a corresponding increase in the number of individual tests, since all the markers are screened in parallel in a single procedure. By allowing much smaller sets of related individuals to be used for linkage mapping, the affected- relative-pair and homozygosity-by-descent mapping methods

can greatly reduce the cost and labor involved in developing the human genetic map. Genomic mismatch scanning allows for the practical application of linkage mapping to genetically heterogeneous or quantitative traits, such as cardiovascular disease, asthma, psychiatric disorders, epilepsy, obesity, cancer and diabetes.

The subject methodology does not rely on any previously-mapped genetic markers. Thus, one can use the subject methodology to begin immediately to develop the genetic and physical maps of a genome for which little or no prior map information is available. This can find particularly important application in the breeding of plant or animal species, as well as in development of the genetics of such species.

Each pair-wise analysis allows sites of meiotic recombination to be mapped. In grandparent-grandchild pairs, identity-by-descent maps specifically identify the sites of meiotic recombination in the corresponding parent. ~~Questions such as the relationship between the~~ genetic and physical map, locations of sites of enhanced or diminished recombination, effects of age, sex and other factors on the frequency and distribution of meiotic recombination events, and the relationship between recombination and non-dysjunction can be readily investigated in this way.

Finally, the ability to detect directly regions of the genome that have lost heterozygosity may be useful in identifying putative tumor-suppressor genes and in the earlier diagnosis of malignancies, since loss of heterozygosity at specific loci appears to be an important genetic event in the development of many cancers.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of 5 the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for separating DNA duplexes capable of being used for genetic mapping or identification, from a complex mixture of DNA from two related sources, wherein
5 each of said sources contributes at least about 5×10^4 bp of DNA, said method comprising:

digesting the DNA from first and second related sources to provide restriction fragments, wherein said DNA from said first and second related sources is
10 distinguishable as a result of differential modification of said DNA or use of different restriction enzymes in said digesting, to provide DNA duplexes consisting of homohybrids and heterohybrids;

separating homohybrids from heterohybrids;
15 introducing lesions in heterohybrids having mismatches; and

isolating heterohybrids having lesions from perfectly complementary heterohybrids.

2. A method according to Claim 1, wherein said
20 different modification and separating is by means of:
methylating the DNA from one of said related sources or methylating the DNA from both of said sources with different methylases;

cleaving said DNA duplexes with a methyl sensitive
25 restriction enzyme resulting in cleaving of homohybrid DNA; and
segregating heterohybrid DNA from cleaved homohybrid DNA;

and said introducing lesions is by means of:
30 bringing together said heterohybrid DNA with enzymes of the methyl-directed mismatch repair pathway and an exonuclease, whereby nicks are introduced into said heterohybrid DNA comprising a lesion and said lesion is extended into a gap by said exonuclease to provide
35 partially single stranded and single stranded DNA;

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dividing said partially single-stranded and single stranded DNA from said perfectly complementary DNA duplexes; and

labeling said perfectly complementary DNA duplexes
5 for use in genetic mapping or identification.

3. A method according to Claim 2, wherein said enzymes comprise MutL, MutS, and MutH of *E. coli*.

4. A method according to Claim 2, wherein included in said combining step is helicase II, single-strand
10 binding protein, and at least one of exonuclease I and VII; or wherein said exonuclease is exonuclease III of *E. coli*.

5. A method according to Claim 2, wherein said dividing comprises:
15 combining said partially single-stranded and single stranded DNA and DNA duplexes with benzoylated naphthylated DEAE cellulose (BNDC) at high salt concentration; and

freeing the DNA bound to the BNDC cellulose from the
20 perfectly complementary DNA duplexes.

6. A method for separating DNA duplexes capable of being used for genetic mapping or identification, from a complex mixture of DNA from two related genomes, wherein each of said genomes contributes at least about 10^6 bp of
25 DNA, said method comprising:

combining DNA restriction fragments from first and second related genomes, wherein said DNA substantially consists of restriction fragments comprising a GATC sequence, under melting and reannealing conditions to form
30 homohybrid and heterohybrid DNA duplexes, wherein said DNA fragments from said first and second sources are different;

segregating homohybrid from heterohybrid DNA duplexes by means of the difference in said DNA duplexes;

bringing together heterohybrid DNA duplexes with enzymes consisting of MutL, MutS, and MutH of *E. coli*, resulting in lesions consisting of nicks in DNA duplexes that contain mismatches;

- 5 treating the resulting mixture of nicked and unnicked DNA molecules with exonuclease III, such that nicked DNA molecules in the mixture are rendered partially single stranded; and

10 separating said partially single stranded DNA from completely double stranded DNA.

7. A method according to Claim 6, wherein said method comprises one of:

- including in said bringing together, or in a subsequent step, a DNA polymerase, and labeled
15 nucleotides, wherein said labeled nucleotides become incorporated into said nicked DNA duplexes; and

separating said labeled DNA from unlabeled DNA by means of said label; or

- ~~combining said partially single stranded and single~~
20 stranded DNA and completely double stranded DNA with benzoylated naphthylated DEAE cellulose at high salt concentration; and

separating the DNA bound to the cellulose from the completely double stranded DNA; or

- 25 cleaving partially single stranded DNA at the site of said single strand to provide small DNA duplexes; and separating said small DNA duplexes from uncleaved DNA duplexes.

8. A method according to Claim 6, wherein said DNA
30 restriction fragments are different in having different termini, wherein the difference in termini is used to separate heterohybrids from homohybrids.

9. A method according to Claim 6, wherein said bringing together further comprises:

including with said heterohybrid DNA, helicase II, exonuclease I and/or VII, single strand binding protein, DNA polymerase III, and labeled nucleotides, wherein said labeled nucleotides become incorporated into partially single stranded DNA to provide labeled DNA; and

separating said labeled DNA from unlabeled DNA by means of said label.

10. A method for identifying nucleic acid areas of identity with a probe obtained by a separating method for separating DNA duplexes from a complex mixture of DNA from two related sources, wherein each of said sources contributes at least about 5×10^4 bp of DNA, said method comprising:

digesting the DNA from first and second related sources to provide restriction fragments, wherein said DNA from said first and second related sources is distinguishable as a result of differential modification of said DNA or use of different restriction enzymes in said digesting, to provide DNA duplexes consisting of homohybrids and heterohybrids;

separating homohybrids from heterohybrids; introducing lesions in heterohybrids having mismatches; and

isolating heterohybrids having lesions from perfectly complementary heterohybrids; and

labeling said perfectly complementary heterohybrids; said method comprising:

combining said probe with an ordered array of DNA molecules representing at least a portion of a genetic map under conditions wherein said probe hybridizes to homologous DNA; and

detecting the areas of identity by means of said label.

11. A method according to Claim 10, wherein said ordered array is a metaphase chromosome spread.

12. A method according to Claim 10, wherein said ordered array is an array of clones representing at least
5 a portion of said genetic map.

13. A method according to Claim 10, wherein said ordered array is an array of DNA sequences amplified *in vitro*, representing at least a portion of said genetic map.

10 14. A method for identifying DNA sequences which are heterozygous for the same locus in the genome of an individual, said method comprising:

digesting genomic DNA with at least one restriction enzyme to provide small DNA fragments of from about 200 to
15 5000 bp;

melting and reannealing said DNA fragments;
~~combining said DNA fragments with enzymes of the~~
methyl-directed mismatch repair pathway, whereby nicks are introduced into the mismatch-containing fragments of said
20 DNA;

labeling said nicked DNA fragments to provide probes;
combining said probes with an ordered array of DNA molecules representing at least a portion of said genome /
under conditions wherein said probe hybridizes to
25 homologous DNA; and

identifying said DNA sequences by means of said label.

15. A method according to Claim 14, wherein said label comprises incubating said lesion containing small
30 DNA fragments with a polymerase which lacks 3' to 5' exonuclease activity and labeled nucleotide triphosphates.

16. A method for identifying DNA sequences which are heterozygous for the same locus in the genome of an individual, said method comprising:

digesting genomic DNA with at least one restriction
5 enzyme to provide small DNA fragments of from about 200 to 5000 bp;

melting and reannealing said DNA fragments;

combining said DNA fragments with enzymes of the
methyln-directed mismatch repair pathway, whereby nicks are
10 introduced into the mismatch containing fragments of said DNA;

treating said DNA fragments with an exonuclease such
that those fragments with nicks are rendered single-
stranded or partially single-stranded;

15 separating single-stranded and partially single-
stranded fragments from completely double-stranded DNA
fragments by adsorption to BNDC;

labeling said single stranded or partially single-
stranded small DNA fragments or labeling perfectly
20 complementary dsDNA to provide probes;

combining said probes with an ordered array of DNA
molecules representing at least a portion of said genome
under conditions wherein said probe hybridizes to
homologous DNA; and

25 identifying said DNA sequences by means of said
label.

17. A method according to Claim 16, wherein said
labeling of said lesion containing partially single
stranded DNA comprises adding DNA polymerase III and
30 nucleotide triphosphates including a label to said
partially single stranded DNA.

18. A method of genetic mapping comprising:

combining fragmented first genomic DNA from two
different sources under conditions wherein said fragments
35 can anneal together to form heterohybrid dsDNA duplexes
and homohybrid dsDNA duplexes, wherein the size of the

- fragments is selected to substantially ensure that heterohybrids formed from genetic regions which are not identical by descent will contain at least one base mismatch and to ensure the ability to separate perfectly
- 5 matched duplexes from mismatched duplexes;
- separating heterohybrid duplexes from homohybrid duplexes and matched duplexes from mismatched duplexes by preferentially modifying heterohybrid duplexes and mismatched homohybrid duplexes;
- 10 labeling at least one of the strands of said matched heterohybrid duplexes to provide detectable probes without expansion of said matched heterohybrid duplexes; and
- combining second genomic DNA with said detectable probes to detect and/or map sites of matched DNA of said
- 15 sources.

19. A method according to Claim 18, wherein said method further comprises:

- methylating the DNA from at least one of said sources
- ~~at specific consensus sequences with a different enzyme~~
- 20 for the DNA from each source; and
- said separating comprises:
- cleaving said duplexes with at least one restriction endonuclease, wherein duplexes which are unmethylated or doubly methylated are cleaved to provide smaller
- 25 fragments;
- isolating the uncleaved duplexes free of said cleaved duplexes;
- nicking mismatched duplexes and introducing gaps in said nicked mismatched duplexes while leaving matched
- 30 duplexes unchanged; and
- segregating gapped duplexes from matched duplexes.

20. A method according to Claim 19, wherein said nicking and introducing gaps employs the proteins of a methyl directed mismatch repair pathway.

21. A method according to Claim 19, wherein said nicking and introducing gaps employs the proteins of the methyl-directed mismatch repair pathway and exonuclease III.

5 22. A method according to Claim 19, wherein said segregating comprises combining said duplexes with BNDC cellulose and separating DNA bound to said BNDC cellulose from unbound duplexes.

10 23. A method according to Claim 18, wherein said second genomic DNA is an ordered array of DNA molecules representing at least a portion of a genetic map.

24. A kit comprising MutL, MutS, MutH enzymes, labeled triphosphates or labeled linkers, and a DNA polymerase.

15 25. A kit according to Claim 24, further comprising at least one of the following: a modification enzyme, and an enzyme that cleaves at other than hemimethylated sites recognized by said modification enzyme; an exonuclease; helicase II; single-strand binding protein; BNDC
20 cellulose; an ordered array of DNA molecules comprising at least a portion of a genetic map.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/04160

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) C12Q 1/68; C12P 19/34

US CL :435/6, 91; 935/77, 78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91; 935/77, 78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nucleic Acids Research, Vol. 14, Number 18, issued 1986, CASNA et al., "Genomic Analysis II: Isolation of High Molecular Weight Heteroduplex DNA Following Differential Methylase Protection and Formamide-PERT Hybridization", pages 7285-7303; see pages 7289-7296 and pages 7286-7287.	1-5, 10-15, 18-23
Y	Nucleic Acids Research, Vol. 14, Number 18, issued 1986, SANDA, et al., "Genomic Analysis I: Inheritance Units and Genetic Selection in the Rapid Discovery of Locus Linked DNA Markers", pages 7265-7283, see pages 7266, 7268-7271 and 7281.	1-23

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* "A"	document defining the general state of the art which is not considered to be part of particular relevance	* "T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* "E"	earlier document published on or after the international filing date	* "X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* "L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* "Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* "O"	document referring to an oral disclosure, use, exhibition or other means	* "&"	document member of the same patent family
* "P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENCE, Vol. 245, issued 14 JULY 1989, LAHUE et al., "DNA Mismatch Correction in a Defined System", pages 160-164, see entire document.	2-6, 14-22, 24
Y	FEBS LETTERS, Vol. 228, Number 2, issued FEBRUARY 1988, NORRIS et al., "Sizing of Single-Stranded Regions in Double-Stranded DNA by Preparative Benzoylated DEAE-Cellulose Chromatography", pages 223-227; see entire document.	6, 16, 17, 22, 24, 25
Y	T MANIATIS et al., MOLECULAR CLONING, A LABORATORY MANUAL, published 1982 by Cold Spring Harbor Laboratory (New York), 545 pages; see pages 143, 108, 109.	6-9, 15-17

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